

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A01H 5/00, 1/00, 1/04, C12N 15/05, 15/09, 15/11	A1	(11) International Publication Number: WO 99/05903 (43) International Publication Date: 11 February 1999 (11.02.99)
(21) International Application Number: PCT/US98/15910 (22) International Filing Date: 27 July 1998 (27.07.98) (30) Priority Data: 60/054,267 30 July 1997 (30.07.97) US (71) Applicant (for all designated States except US): WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 Walnut Street, Madison, WI 53705 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HELGESON, John, P. [US/US]; 809 Oneida Place, Madison, WI 53711 (US). AUSTIN, Sandra [US/US]; 2337 Fox Avenue, Madison, WI 53711 (US). NAESS, Sara, K. [US/US]; 2586 Prairie Ridge Road, Verona, WI 53593 (US). (74) Agents: REED, Janet, E. et al.; Dann Dorfman Herrell & Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103-2307 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: GERMPLASM AND MOLECULAR MARKERS FOR DISEASE RESISTANCE IN POTATO		
(57) Abstract <p>This invention provides novel germplasm, breeding stock, genetic markers and methods for introducing resistance to late blight and other diseases into cultivated potato plants. The germplasm is derived from a segment of the <i>Solanum bulbocastanum</i> genome that contains one or more genes conferring resistance to late blight. The <i>S. bulbocastanum</i> genome is introduced into cultivated potato by somatic hybridization of <i>S. bulbocastanum</i> with <i>S. tuberosum</i>. Also provided is a <i>S. bulbocastanum</i> RAPD fragment marker that co-segregates with the disease resistance gene. In addition, chromosome 8 RFLP markers are provided, which also are tightly linked to the resistance gene(s). These markers are used to monitor passage of the disease resistance trait in breeding crosses. The markers are also used to facilitate isolation of the <i>S. bulbocastanum</i> gene or genes responsible for the disease resistance.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

**GERMPLASM AND MOLECULAR MARKERS FOR
DISEASE RESISTANCE IN POTATO**

This application claims priority to U.S. Provisional Application Serial No. 60/054,267, filed July 30, 1997, which is incorporated by reference herein in its entirety.

5 Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described herein, which was made in part with funds from the United States Department of Agriculture.

10

FIELD OF THE INVENTION

 This invention relates to the field of genetic manipulation of higher plants. More specifically, the invention relates to novel germplasms, breeding stocks
15 and molecular markers created or identified by somatic fusion of domestic and wild potato species, which are useful for development of potato varieties resistant to late blight and other fungal pathogens.

20 **BACKGROUND OF THE INVENTION**

 Several publications are referenced in this application in parentheses in order to more fully describe the state of the art to which this invention
 pertains. Full citations for these references are found
25 at the end of the specification. The disclosure of each of these publications is incorporated by reference herein.

 Plant diseases cause billions of dollars in losses to farmers in the United States and elsewhere in
30 the world every year. Generating crop plants that are naturally resistant to disease has been a goal of plant breeders for decades. Classical breeding methods have been supplemented in recent years by molecular genetic

techniques, e.g., to identify a gene that encodes a protein with antifungal or antibacterial properties (often not a plant gene) and then express this gene at high levels in a plant.

5 Another approach is to use genes from wild species to improve disease resistance and other agronomic characteristics of cultivated crops. For the most part, however, breeders have been restricted to those genetic combinations that can be obtained by direct sexual
10 crosses or through bridging crosses through several species.

 Protoplast fusion in some cases has provided a wider range of available genes. By this technique, the somatic cells of two species are combined. Plants can
15 then be regenerated from the combinations and examined for the expression of the desired attributes and for fertility. In this manner it is possible to incorporate useful traits from widely separated, sexually incompatible, species into breeding lines. Using other
20 molecular techniques, it is also possible to identify genes in those wild species responsible for conferring the useful trait, such as resistance to one or more plant pathogens. The genes can then be incorporated into the genomes of a variety of different species to develop
25 resistance to one or more plant pathogens.

 Potato (*Solanum tuberosum*) is the world's fourth most valuable crop. In the United States, the value of the crop exceeds two billion dollars each year. In spite of its high value, the commercial crops are subject to
30 many disease problems, including foliar diseases such as late blight and early blight, virus diseases, soil problems such as those caused by nematodes or *Verticillium* species, and bacterial diseases such as bacterial wilt (in the field) or *Erwinia* soft rot (in
35 storage). These diseases are costly in terms of crop loss, expenses associated with application of chemicals and environmental impact of pesticide use. Such costs

could be minimized or avoided if resistant potato varieties were available. However, adequate resistance for late blight, *Erwinia* soft rot and many other diseases has not been incorporated into potato cultivars, partly because of the lack of a good diversity of resistance genes that breeders can use to develop resistant cultivars.

Of particular importance is the late blight disease, cause by the fungus *Phytophthora infestans*. Late blight remains one of the most devastating diseases of potatoes worldwide. Despite its importance, no major cultivars with adequate late blight resistance are grown in the United states today. Until recently, crops were protected from the disease by cultural methods (e.g. crop rotation, crop hygiene) and with fungicides. The absence of compatible mating types within the U.S. heretofore has prevented sexual recombination. However, a second mating type has now become widespread in the U.S. and many lines of the fungus have become resistant to one of the key, very effective, systemic fungicides (Metalaxyl) registered for potato late blight control.

The late blight fungus is also a devastating pathogen on crops other than potato. It infects tomatoes, eggplants and other solanaceous species. Other *Phytophthora* species are pathogenic to a wide array of agronomically important plants, including grapes, avocados and several varieties of fruit and nut trees. Accordingly, a source of resistance to *Phytophthora* species that could be introduced into these species by molecular genetic techniques would also be of great value.

Possible sources of resistance to many potato pathogens exist in related *Solanum* species. Several *Solanum* species have been crossed with the cultivated potato in an effort to introgress disease-resistance genes.

When sexual crossing techniques have failed,

disease resistance transfers have been attempted with somatic cell fusions. Potato protoplasts have been fused with a number of sexually incompatible wild *Solanum* species (e.g., *S. brevidens*, *S. bulbocastanum*, *S.*
5 *commersonii*, *S. polyadenium*, *S. etuberosum*), and many fertile somatic hybrids have been regenerated (Austin et al., 1985, 1993; Ehlenfeldt & Helgeson, 1987; Kim-Lee et al., 1993; Novy & Helgeson, 1994b). Somatic hybrids have been screened for useful disease resistance (Helgeson et
10 al., 1986; Austin et al., 1988; Novy & Helgeson, 1994b) and these resistances are heritable (Helgeson et al., 1993).

Solanum bulbocastanum is a particularly desirable wild species from which to seek useful disease
15 resistance genetic traits, inasmuch as it exhibits resistance to several potato pathogens, including nematodes, early blight, late blight and *Verticillium*. Disease- or pest-resistant somatic hybrids of *S. bulbocastanum* and cultivated potato have been produced by
20 the present inventors and by others. In one instance, analysis of BC1 and BC2 progeny of a nematode-resistant *S. bulbocastanum* - *S. tuberosum* somatic hybrid revealed that the nematode resistance locus is likely to reside on chromosome 11 of *S. bulbocastanum* (Brown et al., 1996).
25 However, the chromosomal location of late blight resistance in *S. bulbocastanum* heretofore has not been identified.

Clearly, there is an ongoing need to identify such resistances in wild potato species, such as *S.*
30 *bulbocastanum*, to continue improving the disease resistant characteristics of cultivated potatoes. A need also exists to identify the genes in wild potato species that are responsible for disease resistance. Once isolated, these genes can then be introduced by molecular
35 genetic techniques into species other than potato to confer resistance to one or more plant pathogens.

SUMMARY OF THE INVENTION

This invention provides novel germplasms, breeding stocks, molecular markers and methods for introducing late blight resistance into cultivated potato plants. The invention further provides genomic DNA segments from *S. bulbocastanum* useful for introducing resistance to the late blight fungus, *Phytophthora infestans*, into species other than potato.

According to one aspect of the invention, a potato germplasm is provided that confers resistance to the late blight fungus, *Phytophthora infestans*, as well as other fungal pathogens, including early blight, *Erwinia* soft rot and *Verticillium*. The most fundamental form of this germplasm is a tissue culture produced by somatic hybridization of *S. tuberosum* with *S. bulbocastanum*. Fertile plants regenerated from these hybrids are also provided, along with progeny resulting from crosses with agronomically preferable cultivated potato species.

According to another aspect of the invention, a late blight-resistant potato plant is provided, comprising a segment of a genome from *Solanum bulbocastanum* which contains a gene that confers resistance to late blight. In a preferred embodiment, the genomic segment of *S. bulbocastanum* is from chromosome 8, and co-segregates with one or more of the following markers: (1) a RAPD marker referred to herein as GO2₅₈₆; (2) a RAPD marker referred to herein as PO9₅₈₇; and (3) RFLP marker CT88, RFLP marker, RFLP marker CT148, RFLP marker CT252 and RFLP marker CT68. The potato plant may also be resistant to at least one additional disease, such as potato early blight, *Erwinia* soft rot, and *Verticillium* wilt.

In a preferred embodiment, the aforementioned late blight resistance gene is incorporated into the potato plant by somatic hybridization between a cell of a parent of the plant and a cell of *Solanum bulbocastanum*.

In another embodiment, the late blight resistance gene is incorporated into the plant by genetic transformation of a cell of the plant with a plant transformation vector containing the gene.

5 According to another aspect of the invention, an isolated nucleic acid molecule is provided, which is complementary to part or all of a 0.6 kb segment of a *Solanum bulbocastanum* genome, which co-segregates with a gene that confers resistance to late blight. In a
10 preferred embodiment, the segment comprises part or all of the RAPD marker GO2₅₈₆ or PO9₅₈₇, having the sequence of SEQ ID NO:1 or SEQ ID NO:2, respectively.

 According to another aspect of the invention, a method of monitoring late blight resistance in a breeding
15 cross of progeny of a fertile somatic hybrid of *Solanum tuberosum* and *Solanum bulbocastanum* is provided. The method comprises: (a) performing the cross; (b) isolating genomic DNA from individual progeny of the cross; and (c) detecting in the genomic DNA the presence
20 or absence of a genetic marker that is pre-determined to co-segregate with the late blight resistance, the presence or absence of the marker being indicative of the presence or absence of the late blight resistance in the individual progeny of the breeding cross. In a preferred
25 embodiment, the marker is selected from the group of RAPD and RFLP markers listed above.

 According to yet another aspect of the invention, a method of identifying a *Solanum*
bulbocastanum gene that confers resistance to late blight
30 is provided. The method comprises: (a) cloning a DNA segment that co-segregates with the late blight resistance phenotype in progeny of somatic hybrids of *Solanum bulbocastanum* and *Solanum tuberosum*; (b) providing a genomic library of the *Solanum bulbocastanum*
35 genome; (c) isolating clones of the genomic library that contain segments which hybridize with the co-segregating DNA segment; and (d) identifying at least one gene

disposed within the isolated genomic clones that confers the late blight resistance. In a preferred embodiment, the cloned DNA segment that co-segregates with late blight resistance comprises part or all of one of the

5 RAPD or RFLP markers listed above.

According to further aspects of the invention, a late blight resistance gene from *Solanum bulbocastanum*, produced by the aforementioned method, is provided. Also provided is a transgenic plant comprising the resistance

10 gene.

The advantages of the present invention will be better understood in view of the detailed description and examples set forth below.

15 BRIEF DESCRIPTION OF THE DRAWING

Figure 1. RFLP analysis of somatic hybrids between *S. bulbocastanum* and potato. The probe used in the analysis was TG310, a tomato genomic probe specific for Chromosome 1 of tomato and potato.

20 Figure 2. MapMaker analysis of chromosome 8 of *Solanum bulbocastanum* with RAPD (randomly amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) markers and resistance to late blight. Percentages in parentheses (left column) indicate

25 recombination frequencies calculated by dividing the deviation from complete co-inheritance by the population size. Numbers to the right of the percentage column indicate distances between markers in centimorgans. Numbers in parentheses to the right of the chromosome 8

30 diagram represent the arbitrary code number of the individual in the population. The far right column lists RAPD markers, which are named by the decameric primer and the size of the amplified transcript (e.g. "G02-586" or "P09-587") and RFLP markers. The resistance locus is

35 indicated by "R". Map scale is 10.0 cM per 1.21 cm.

Figure 3. Tomato chromosome 8 map, after Tanksley et al., 1992, showing approximate corresponding

location of *S. bulbocastanum* late blight resistance gene(s) on tomato chromosome 8.

DETAILED DESCRIPTION OF THE INVENTION

5 In accordance with the present invention, genetic markers have been identified that are linked to late blight resistance derived from somatic hybrids of *Solanum bulbocastanum* and cultivated potato (*Solanum tuberosum*). Backcross (BC₂) populations were analyzed for
10 resistance to late blight and for randomly amplified polymorphic DNA (RAPD) and RFLP markers. Three test populations were derived from two different somatic hybrids between potato and *S. bulbocastanum*. Each somatic hybrid was crossed with the cultivar Katahdin to
15 generate BC₁ parents, all of which were resistant to late blight. BC₁ progeny were crossed with three different potato breeding lines (Norland, Atlantic and A 89804-7), all of which are sensitive to the late blight fungus. Each BC₂ population contained more than 50 individuals and
20 segregated for resistance to late blight. In each population, late blight resistance was correlated (>95%) with the presence of a RAPD marker ("GO2₅₈₆") keyed to chromosome 8 of *S. bulbocastanum*. The identification of this novel molecular marker is described in greater
25 detail in Example 2.

 Further genetic analysis has led to the identification of another RAPD marker and several RFLP markers that are also associated with the resistance gene or genes of *S. bulbocastanum* chromosome 8. These
30 include, for example, RFLP marker CT88 and RAPD marker PO9₅₈₇, which appear to flank the resistance locus (see Figure 2). Other closely linked RFLPs include CT148, CT252 and CT68 (Figure 2). Together, these markers define a specific location on *S. bulbocastanum* chromosome
35 8 that carries the gene(s) which confer resistance to late blight and certain other potato diseases, as discussed in greater detail herein. The approximate

location of the resistance genes on Chromosome 8 (as mapped by Tanksley et al., 1992) is shown in Figure 3.

This invention provides a new and useful germplasm and breeding stock for breeding potato
5 cultivars with resistance to late blight. The germplasm comprises fertile hybrids produced by somatic fusion of *S. bulbocastanum* with *S. tuberosum*, and progeny thereof, which contain that portion of the *S. bulbocastanum* genome carrying the late blight resistance gene or genes. The
10 presence of this genomic fragment is conveniently monitored by the presence of closely linked RAPD markers, GO2₅₈₆ or PO9₅₈₇, or closely-linked RFLP markers, such as CT88, as described in greater detail below. Particularly preferred breeding stock is obtained by repeated
15 backcrosses of the somatic hybrid with potato cultivars having desirable agronomic qualities, with the presence of the late blight resistance-conferring genomic segment of *S. bulbocastanum* being monitored by detection of one or more of the relevant RAPD or RFLP markers.

20 Creation of *S. tuberosum* - *S. bulbocastanum* somatic hybrids, selection of fertile, resistant plants, and production of subsequent backcross generations comprising the disease resistance gene or genes are all accomplished by methods well known to plant breeders and
25 molecular biologists. Preferred methods are described in greater detail in Example 1.

This invention also provides novel molecular markers to facilitate selection of breeding progeny that contain the resistance-conferring genomic segment from *S.*
30 *bulbocastanum*, without having to perform field or greenhouse trials for disease resistance. One closely linked marker, GO2₅₈₆, was created through the use of RAPD markers, using commercially available oligonucleotide 10-mers as primers for PCR amplification. RAPD markers are
35 generated by incubating genomic DNA with a population of 10-mers under conditions that allow the oligonucleotides to bind to any complementary sequences in the genomic

DNA. The length of DNA between two bound sets of primers is amplified by PCR, thereby generating a DNA segment which is a copy of the segment between the primers in the genomic DNA, i.e. a RAPD fragment. The nucleotide
5 sequence of the GO2₅₈₆ RAPD marker is set forth herein as SEQ ID NO:1.

RAPD fragments thus created are species specific markers which can be keyed to particular chromosomes by comparative RFLP analysis and can be
10 followed as dominant markers through various crosses. In accordance with the present invention, RAPD marker GO2₅₈₆ (which is a 586 bp fragment primed by the "GO2" decameric oligonucleotide purchased from Operon Technologies, Inc.) was keyed to *S. bulbocastanum* chromosome 8. Analysis of
15 BC₂ progeny of potato-*S. bulbocastanum* somatic hybrids demonstrated that late blight resistance correlates with greater than 95% frequency with the presence of the GO2₅₈₆ RAPD fragment. This tight segregation of late blight resistance with the marker indicates that the resistance
20 gene or genes reside at or near the location of the marker. Accordingly, late blight resistance in further crosses can be monitored by detecting the presence or absence of the marker. In this manner, progeny having a high probability of carrying the resistance gene(s) can
25 be selected without lengthy greenhouse or field trials for disease resistance, thus making the breeding process faster and more efficient.

This invention also provides a second RAPD marker, PO9₅₈₇, which is also closely linked with the
30 resistance gene(s) in *S. bulbocastanum*, and which was identified by the same protocol as set forth above for GO2₅₈₆. The nucleotide sequence of RAPD marker PO9₅₈₇ is set forth herein as SEQ ID NO:2.

This invention further provides RFLP molecular
35 markers, useful in facilitating selection of breeding progeny that contain the resistance-conferring segment of *S. bulbocastanum*. These markers can also assist in

defining the location of the resistance genes on the chromosome, and obtaining isolated genomic segments containing the gene(s). The nucleotide sequence of RFLP CT88 from three different sources (published by Tanksley
5 et al. (<http://probe.nalusda.gov:8300/cgi-bin/browse/solgenes>), from R4 potato, and from *S. bulbocastanum*) are set forth herein as SEQ ID NOS: 3, 4 and 5 respectively).

Once created, useful RAPD or RFLP fragments can
10 be maintained in any suitable cloning vector. For instance, the GO2₅₈₆ marker generated in accordance with the present invention is maintained in a plasmid vector provided with a commercially available PCR kit (Invitrogen, Inc.). It is noted that obtaining RAPD
15 should be replicable by anyone of skill in the art, using the commercially available decamers and the methods described in Example 2 and references cited therein. Alternatively, RAPD fragments described herein can be duplicated by nucleotide synthesis using standard
20 methodologies.

The RAPD fragments, or portions thereof, or any of the linked RFLPs discussed above or shown in Figure 2, are used to monitor the presence or absence of the late blight resistance gene(s) by labeling them as probes to
25 hybridize with complementary sequences in the *S. bulbocastanum* genome. The cloned fragments may be labeled according to any standard means, many of which are set forth in "Current Protocols in Molecular Biology", eds. Frederick M. Ausubel et al., John Wiley & Sons, 1997. The complementary genomic DNA is detected by
30 one of several standard methods including, but not limited to, (1) *in situ* hybridization; (2) Southern hybridization (3) "dot blot" hybridization; and (4) assorted amplification reactions such as polymerase chain
35 reactions (PCR). Detection of the complementary genomic DNA is indicative of the presence of the late blight resistance-conferring gene(s), due to the close linkage

of the R gene(s) and the RAPD marker or RFLPs, as discussed above.

The RAPD fragments, or portions thereof, or any of the aforementioned RFLP fragments, may also be used to
5 identify and isolate the closely-linked late blight resistance gene of *S. bulbocastanum*, using methods well known to molecular biologists. In a preferred embodiment, a genomic library of *S. bulbocastanum* is constructed a suitable cloning vehicle, e.g. cosmid,
10 yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC). The library is then screened by hybridization with the cloned RAPD fragment and/or one or more of the RFLP markers, and hybridizing clones are isolated. The hybridizing clones are then further
15 analyzed, e.g. by mapping, sequencing and transcript analysis, to identify candidate open reading frames that may encode the resistance factor(s). Once candidate open reading frames are identified, they in turn may be further analyzed (e.g. by construction and *in vitro*
20 expression of a cDNA molecule) in order to characterize the resistance gene and its encoded protein.

The disease resistance-conferring clone thus identified may be used to introduce late blight resistance into cultivated potato by molecular genetic
25 techniques. For example, a binary bacterial artificial chromosome (BIBAC) vector may be used to mobilize a BAC genomic insert into potato (the vector BIBAC2 (Hamilton et al, 1996) has been used to mobilize large (>150 kb) DNA inserts into tobacco). A BIBAC vector containing the
30 relevant genomic insert from *S. bulbocastanum* can be transferred into an *Agrobacterium tumefaciens* and used to transform selected potato cultivars. Alternatively, potato cells can be transformed via biolistic delivery of the BIBAC clone. Putative transgenic clones can then be
35 evaluated by standard methods to determine if the transformation has been successful.

The disease resistance-conferring clone also

may be used to introduce resistance to *Phytophthora infestans* in species other than potato, which are sensitive to infection by that organism. These species include, but are not limited to, tomato, eggplant and other *Solanum* species. Furthermore, it is possible that the late blight resistance gene(s) of *S. bulbocastanum* may confer resistance to diseases other than late blight, and so may be of even broader utility for introducing disease resistance in potato and other plant species. The gene may be particularly useful for introducing resistance to other *Phytophthora* species pathogenic to grapes, avocados, fruit trees and nut trees. Moreover, once the gene's function has been determined, this may lead to the discernment of new mechanisms of resistance in other species.

The following examples are provided to describe the invention in greater detail. They are intended to exemplify, not to limit, the scope of this invention.

20

EXAMPLE 1

Resistance to Late Blight in Somatic Hybrids of *Solanum bulbocastanum* and Potato, and Progeny Thereof

A Mexican wild species, *S. bulbocastanum*, is highly resistant to late blight. However, *S. bulbocastanum* is a 1EBN species and thus extremely difficult to cross directly with potato.

Somatic hybridization can provide a means of bypassing sexual incompatibility between *Solanum* species, leading to fertile plants that can be used directly in breeding programs. The experimental results set forth in this Example demonstrate that the resistance in *S. bulbocastanum* can be captured and passed on to potato breeding lines by the use of somatic hybridization.

MATERIALS AND METHODS

Potato and related species used for somatic hybridization were obtained from Dr. John Bamberg and his

colleagues at the Inter-Regional Potato Introduction Station (NRSP-6), 4312 Highway 42, Sturgeon Bay, WI. These include *S. bulbocastanum*, PI 243510 and *S. tuberosum* PI 23900 (potato). Elite copies of potato cultivars (Katahdin, Atlantic) were obtained from the Wisconsin potato certification program. All cultivars and wild species, and test materials were routinely maintained clonally *in vitro* as described by Haberlach et al (1985). The individual clones were multiplied *in vitro* for analyses.

Protoplasts were isolated from leaves of *in vitro* shoots as described by Haberlach et al. (1985). Somatic hybridizations with the protoplasts were performed using a polyethylene glycol (PEG) protocol. For the most part, the procedure of Austin et al. (1985) was followed. However, after PEG additions, dilutions and pelleting of the cells after the fusion attempts, the cells were suspended in 0.3 M sucrose rather than 0.6 M mannitol. The cell suspension was gently shaken (40 RPM) for 45 minutes and then centrifuged (HNII centrifuge, IEC) at 1300 rpm for 10 minutes in a Babcock bottle. This modification resulted in viable protoplasts and fused cells being concentrated at the surface of the sucrose solution in the bottle, thus separating the viable cells from the pelleted debris.

The resulting fused cells were regenerated into whole plants in a manner similar to that reported by Haberlach et al. (1985). Initially, the cells were plated onto culture medium (CUL, Haberlach et al. 1985) and, after macroscopic calli had appeared, the calli were transferred to differentiation medium (DIF, Haberlach et al. 1985). After 2-3 weeks, the calli were transferred to the differentiation medium developed by Lam (1977). After buds had formed, the calli were transferred to proliferation medium (PM, Haberlach et al. 1985). Shoots that formed were then excised and rooted on standard propagation medium (PROP, Haberlach et al.

1985) and maintained in test tubes *in vitro*. Clonal copies of the reference copy were made for experiments.

DNA extractions and restriction fragment length polymorphism (RFLP) analyses were carried out as described by Williams et al. (1990). Chromosome specific tomato genomic (TG) and cDNA (CD) probes were obtained from Dr. Steve Tanksley, Cornell Univ. Four putative somatic hybrids were analyzed by this method. To complete the analyses for hybridity, randomly amplified polymorphic DNA (RAPD) analyses were carried out as described in greater detail in Example 2. A total of 109 primers (from 380 primers tested) were selected that give clearly scorable polymorphisms between potato and *S. bulbocastanum*. Several of these were used with each of the putative somatic hybrids.

Three of the hybrids have been used extensively in further experiments. These were designated J101, J103, and J138. In crosses, the potato parent was designated with K (for Katahdin) or A (for Atlantic). Thus, for example, J101K1 was the first seed germinated from a berry obtained from the cross of J101 and Katahdin. Similarly, J101K6 and J101K27 were the seedlings obtained from seed 6 and seed 27 respectively from that cross. The BC2 progeny were named by adding the seed number and cultivar lines in that cross. Thus the cross designated as J101K6A22 is the 22nd seedling from the cross of line J101K6 with Atlantic. This shortcut avoided use of the longer and less informational term ((*S. bulbocastanum* + *S. tuberosum*) x Katahdin) x Atlantic that could be applied to this individual.

Comparisons were made of susceptible and resistant plants in the field. For these studies, the percentage of leaves showing late blight lesions was recorded at various times during the growing season. For detailed greenhouse studies, a modified Horsfall-Barret rating scheme was used to estimate percent leaf infection by *P. infestans*. The ratings and the ranges of %

infections associated with these scores are as follows:
9, no visible infection; 8, <10%; 7, 11-25%; 6, 26-40%;
5, 41-60%; 4, 61-70%; 3, 71-80%; 2, 81-90% 1, >90%;
0, 100% infection.

5

RESULTS

In total, 80 shoots were obtained from 23
calli. Twenty four of these plants (from 5 calli) showed
obvious morphological differences from either of the
10 parent species. The other 56 plants appeared very
similar to potato. Initially, chromosome-specific
restriction fragment length polymorphism (RFLP) markers
were used to confirm that four of the plants derived
from fusion of *S. bulbocastanum* and potato cells were
15 indeed somatic hybrids. Prominent potato bands were
retained by the hybrids in addition to the diagnostic *S.*
bulbocastanum bands (Figure 1). The rest of the
potentially hybrid plants were evaluated with RAPD probes
(see Example 2). A total of 13 more somatic hybrids
20 were confirmed by these techniques. The confirmed
hybrids were derived from four different callus pieces,
and thus probably from four different fusion events.

The appearance of leaves and stems of the
parent plants and representative somatic hybrids was
25 examined. As has been the case with many of our other
hybrids, traits of both of the parent species could be
seen in the hybrids. In this case, the purple coloration
of the *S. bulbocastanum* stems was expressed in the
hybrids. However, leaves of the hybrids were compound
30 rather than single as was the case of the wild species.

Crosses of four of the somatic hybrids were
undertaken with the potato cultivars "Katahdin" and
"Atlantic" to test for fertility of the hybrids. Each of
the tested hybrids yielded viable seeds and sexual
35 progeny. Further crosses of selected individuals from
these progeny lines were also successful. Thus, parental
lines and two successive backcross populations were

available for evaluation of disease resistance.

Preliminary laboratory tests for resistance to *P. infestans* were made with detached leaves or leaf discs indicated that the somatic hybrids and some of the
5 progeny retained at least some of the resistance to late blight that was shown by the *S. bulbocastanum* parent.

These preliminary laboratory results were confirmed in field tests during a first growing season. Somatic hybrids as well as progeny from somatic hybrids
10 between *S. bulbocastanum* and potato, showed remarkable resistance and were clearly noticeable in test plots as "green islands " in a brown background of dead potato lines. Although the cultivars Atlantic, Russet Burbank, and Snowden were killed, eleven different experimental
15 lines showed less than 10% infection. In each case, the live test plants were surrounded with the susceptible cultivar, Russet Burbank, which had been killed by the fungus by August 9 of that growing season. In contrast, % foliage infections on August 15 were 5.0% and 7.8% for
20 J101K27 and J101K6A22, respectively.

Fourteen BC₁ and BC₂ lines were tested in Toluca, Mexico in a second growing season the following summer. Good resistance in the Toluca field test was also obtained with all lines that were resistant in
25 Wisconsin in the previous summer.

Additional field experiments were carried out at Hancock, Wisconsin, in a third growing season. Again, a severe natural late blight epidemic was obtained in test plots and yields of common cultivars were severely
30 depressed by the late blight epidemic. For example, in plots where an effective fungicide spray regime was maintained, Russet Burbank yields were as high as 1.7 kg/plant. In the yield trial where no fungicide was used, this was cut almost in half to 0.86 kg/plant. One
35 of the *S. bulbocastanum* -derived lines , J103K7, topped all 90 test lines with a yield of 1.36 kg/plant and J138A12 ranked fourth at Hancock in the third growing

season, with a yield of 1.32 kg/plant.

To test the resistance of potentially segregating BC₁ and BC₂ populations, a facility was constructed in the new research greenhouses at the University of Wisconsin Biotron. There, close control of humidity and temperature made uniform epidemics possible. Segregation of resistance and susceptibility was obtained for each of six BC₁ from four different somatic hybrids.

Representative tests from one of these lines are shown in Table 1. Three of these lines were further crossed to Atlantic or Norland. Representative results on these BC₂ lines are included in Table 2. Again, clear segregation of resistance and recovery of both parental extremes of susceptibility and resistance were obtained with these lines.

Table 1. Representative data from Biotron tests of BC₁ lines for late blight resistance

Plant line	Average Blight score		
	5day	8 day	12 day
J101K09	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
J101K27	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
J101K06	8.8 ± 0.5	9.0 ± 0.0	8.8 ± 0.4
J101K10	9.0 ± 0.4	8.8 ± 0.4	8.6 ± 0.9
J101K30	9.0 ± 0.0	9.0 ± 0.0	8.4 ± 0.9
J101K16	8.8 ± 1.0	8.8 ± 0.5	7.8 ± 1.0
J101K25	8.6 ± 1.1	8.4 ± 0.9	8.2 ± 1.1
J101K02	8.6 ± 0.4	8.0 ± 1.2	7.8 ± 0.4
J101K20	8.6 ± 0.7	7.4 ± 0.9	7.0 ± 0.7
J101K33	7.4 ± 1.8	5.4 ± 0.9	6.2 ± 1.8
J101K19	6.8 ± 2.4	5.6 ± 2.7	5.2 ± 2.4
J101K11	6.0 ± 1.3	4.6 ± 1.1	4.2 ± 1.3
J101K12	7.2 ± 2.5	5.2 ± 2.4	3.8 ± 2.5
J101K18	6.8 ± 1.8	4.6 ± 1.3	3.6 ± 1.8
J101K21	5.2 ± 1.3	2.2 ± 1.3	0.6 ± 1.3
<i>S. bulbocastanum</i>	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
Somatic hybrid J101	8.6 ± 0.5	7.8 ± 0.8	8.2 ± 0.0
<i>S. tuberosum</i> PI 203900	7.0 ± 0.0	5.8 ± 1.3	5.6 ± 1.9
<i>S. tuberosum</i> cv "Kathadin"	4.8 ± 0.4	2.0 ± 0.7	1.0 ± 1.2

Table 2. Examples of segregation for late blight resistance in BC₂ progeny of a cross between BC₁ line J101K6 and *S. tuberosum* cv. Atlantic.

5	Plant line	Average blight score		
		7 day	10day	15 day
10	J101K6A21	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
	J101K6A4	8.8 ± 0.4	8.8 ± 0.4	9.0 ± 0.0
	J101K6A22	9.0 ± 0.0	9.0 ± 0.0	8.8 ± 0.4
	J101K6A2	9.0 ± 0.0	9.0 ± 0.0	8.8 ± 0.4
	J101K6A3	5.2 ± 3.1	5.4 ± 3.6	2.0 ± 3.9
	J101K6A10	3.4 ± 2.1	3.0 ± 2.0	1.4 ± 1.9
	J101K6A50	5.6 ± 3.4	3.4 ± 3.6	0.0 ± 0.4
15	J101K6A24	2.6 ± 0.9	1.4 ± 0.5	0.0 ± 0.0
	<i>S. bulbocastanum</i>			
	PI 243510	9.0 ± 0.0	8.8 ± 0.4	9.0 ± 0.0
	<i>S. tuberosum</i>			
20	PI 203900	4.0 ± 1.0	4.0 ± 1.0	0.6 ± 0.9
	J101*	7.8 ± 1.1	8.6 ± 0.5	8.4 ± 0.9
	Kathadin*	4.4 ± 0.5	4.2 ± 0.8	3.4 ± 1.8
	J101K6**	9.0 ± 0.0	9.0 ± 0.0	9.0 ± 0.0
	Atlantic**	3.6 ± 0.5	3.0 ± 0.0	1.8 ± 1.8
25	* Lines crossed to generate BC ₁ lines			
	** Lines crossed to generate BC ₂ lines.			

These results indicate that somatic hybrids between *S. bulbocastanum* and potato are sources of highly effective resistance against late blight. Furthermore, this trait can be passed on to potato breeding lines by conventional sexual crossings. The resistance carries over at least two generations of sexual progeny and, with the third season results just obtained, has been stable for 4 different years in several different locations. As no North American cultivar currently has adequate resistance against this disease, these lines will be very useful for introducing resistance into commercial lines.

The resistance to *P. infestans* from *S. bulbocastanum* appears to be more general than the race-specific resistance derived from *S. demissum* (Black & Gallegly, 1957). Nearly every race of the fungus is known to be found in Toluca, Mexico and was actually isolated from the fields where the progeny of the somatic hybrids showed good resistance. Observations of the

foliage in Toluca in two different growing seasons indicated that some lesions actually formed and that limited sporulation also occurred. Although the disease resistance is highly effective, it is unclear, as yet, the numbers of genes involved. For each of the somatic hybrids tested, the disease resistances of BC₁ lines appear to segregate. Thus, it appears that the late blight resistance gene(s) is (are) heterozygous in the clone of *S. bulbocastanum* utilized in somatic hybridization.

EXAMPLE 2

Identification of RFLPs and a RAPD Marker
Linked to Late Blight Resistance Derived from
Solanum bulbocastanum-Potato Somatic Hybrids

Example 1 described the production of potato-*S. bulbocastanum* somatic hybrids and their progeny, which are resistant to potato late blight. This example describes the identification of DNA markers that co-segregate with disease resistance in these progeny.

Detailed genetic mapping of traits in potato has become possible due to the completion by Bonierbale et al. (1988) of a restriction fragment length polymorphism (RFLP) map in potato. By using tomato clones that had been mapped previously in tomato, a potato map was constructed via a diploid, interspecific cross. The tomato map and the potato map exhibit a nearly collinear gene order (Tanksley et al., 1992).

An alternative method for generating markers is the use of randomly amplified polymorphic DNAs (RAPDs). This technique uses DNA polymerase, synthetic oligonucleotides (10-mers) and genomic DNA mixed together in a PCR thermal cycler to generate bands that are copies of sequences of the genomic DNA present in the mix (J.G.K. Williams et al., 1990). The procedure usually generates between about five and eight specific bands that can be followed, as dominant markers, through various crosses. Moreover, if specific bands can be

linked to a characteristic (such as disease resistance) or to a particular chromosome, or both, the bands can be excised and amplified, then used as a standard RFLP. The method works well to evaluate the extension of DNA
5 introgression from a wild species into a cultivated species. However, RAPDs are species specific, so it is necessary to develop a set for each different species, unlike with RFLPs where inter-species synteny applies.

10 MATERIALS AND METHODS

Somatic hybrids *S. bulbocastanum* (PI 243510) and *S. tuberosum* (PI 203900) were generated by the method of Austin et al. (1985 and 1993), as describe in detail in Example 1. Plants were derived from fertile hybrids,
15 and three lines of these were designated J101, J103 and J138, respectively. The somatic hybrid plants were crossed as the female parent with *S. tuberosum* cv Katahdin (KAT) to give BC₁ progeny. Three BC₁ progeny were crossed as seed parents with three different potato
20 breeding lines (Norland, Atlantic and A 89804-7) to generate BC₂ populations.

For RFLP and RAPD analysis, DNA was isolated exclusively from plants maintained in axenic *in vitro* cultures. DNA manipulations, PCR amplification protocols
25 for RAPD markers (Williams et al, 1990) and assignment of RAPD markers to *S. bulbocastanum* chromosomes by reference to RFLP markers was performed as described by McGrath et al. (1994), with the exception that a modified thermocycle profile was used, as described by McGrath et
30 al. (1996). RAPD markers were named by the deca-nucleotide primer (obtained from Operon Technologies) and the size of the amplified fragment in subscript (e.g., a 586 bp fragment amplified by primer GO2 is represented as GO2₅₈₆).

35 Segregation of RAPD markers was also analyzed with maximum likelihood algorithms contained in the MapMaker computer package (Lander et al., 1987).

MapMaker version 2.0 for the MacIntosh was used. The data were coded and analyzed as a "Haploid" population under the "Data Type" option. The use of MapMaker for the analysis of interspecific somatic hybrids is non-standard and does not provide three-point linkage data. However, recombination frequencies do have meaning in this context. Markers that show identical segregation have a recombination frequency of 0.0%. Markers that deviate from complete co-inheritance by a single change (e.g. a marker is present in one additional individual) show a recombination frequency proportional to the population size; in this instance 1/101 individuals or 1.0%. Thus, multiples of this value indicate the number of differences observed between any pair of markers.

RESULTS

Using RAPD marker analysis, twelve synteny groups were established for *S. bulbocastanum*, corresponding to the base number of chromosomes in that species. The groups were associated with chromosomes by comparative RFLP analysis. A MapMaker analysis of RFLPs and RAPD fragments of synteny group A (chromosome 8) of *S. bulbocastanum* is shown in Figure 2. This synteny group contains the RAPD markers GO2₅₈₆ and PO9₅₈₇ as well as the RFLP CT88. It appears that the resistance area (R) is flanked by CT88 and GO2₅₈₆ on one side and PO9₅₈₇ on the other (Figure 2).

The nucleotide sequence of GO2₅₈₆ is set forth herein as SEQ ID NO: 1. The nucleotide sequence of PO9₅₈₇ is set forth herein as SEQ ID NO:2. Three nucleotide sequences of CT88 are set forth herein. SEQ ID NO:3 is the sequence published by Tanksley et al. (<http://probe.nalusda.gov.8300/cgi-bin/browse/solgenes>); SEQ ID NO:4 is from R4 potato, and SEQ ID NO:5 is from *S. bulbocastanum* (PT29). Slight differences were noted among the three sequences. The R4 potato marker is 589 bp in length, while the *S. bulbocastanum* RFLP is 592 bp

and the Tanksley et al. sequence is 596 bp. In addition, the *S. bulbocastanum* CT88 homolog possesses two TaqI sites, whereas the other two have only one.

The fusion of *S. tuberosum* with *S.*

5 *bulbocastanum* yielded 17 confirmed somatic hybrids. The somatic hybrids are quite resistant to early blight and to late blight. The progeny of some crosses were found to segregate for high resistance to both early blight and late blight. Some of the progeny are highly resistant
10 even to fungal lines that are highly virulent, complex races. Several highly resistant clones were selected and further crossed with potato. Three sets of BC₂ mapping populations have been generated by crosses with potato cultivars. Field studies in Wisconsin first indicated
15 resistance to *Verticillium*, early blight and late blight in these materials. Subsequent studies on late blight resistance were carried out in North Dakota, Prince Edward Island, Washington state, New York, Maine, West Virginia and Toluca, Mexico. The Mexico and Wisconsin
20 studies were repeated for a second and third season and late blight resistance in these materials appears to be durable.

Segregation of late blight resistance in selected BC₁ and BC₂ populations were shown in Example 1,
25 Tables 1 and 2. Table 3 below shows results of segregation analysis of BC₂ progeny with respect to late blight resistance in relation to the presence or absence of RAPD marker G02₅₈₆ for Chromosome 8 of *S. bulbocastanum*. The data shown in Table 3 were generated
30 by PCR amplification, followed by agarose gel electrophoresis and ethidium bromide staining to observe the presence or absence of an amplified 586 bp band.

Table 3. Segregation of BC₂ progeny in relation to the presence or absence of RAPD marker GO2₅₈₆.

5	<u>Clone</u>	<u>Late blight rating</u>			<u>Marker</u>
		<u>7 day</u>	<u>10 day</u>	<u>15 day</u>	
	PI 245310 (BLB)	9.0	8.8	9.0	+
	PI 203900 (TBR)	4.0	4.0	0.6	-
10	J101	7.8	8.6	8.4	+
	Kathadin	4.4	4.2	3.4	-
	J101K6	9.0	9.0	9.0	+
	Atlantic	3.6	3.0	1.8	-
	J101K6A22	9.0	9.0	8.8	+
15	J101K6A50	5.6	3.4	0.0	-
	J101K6A32	8.6	8.4	9.0	+
	J101K6A38	2.8	3.4	1.4	-
	J101K6A21	9.0	9.0	9.0	+
	J101K6A07	6.2	7.0	5.3	-
20	J101K6A12	9.0	9.0	9.0	+
	J101K6A03	5.2	5.4	2.0	-
	J101K6A19	9.0	9.0	8.8	+
	J101K6A18	7.0	7.7	5.3	-
	J101K6A02	9.0	9.0	8.8	+
25	J101K6A54	5.0	3.2	1.0	-

As can be seen from Table 3 (as well as other results not shown), late blight resistance in the BC₂ clones is highly correlated (>95%) with the presence of RAPD marker GO2₅₈₆, which has been keyed to chromosome 8 of *S. bulbocastanum*. This high correlation of the resistance phenotype with the marker indicates that the gene(s) responsible for conferring late blight resistance reside on the chromosome at or near the GO2₅₈₆ marker.

Accordingly, the marker can be used as a molecular tag to follow resistance through breeding programs and to identify and isolate disease-resistant breeding stock. Additionally, knowing the relative proximity of the

resistance gene(s) to the marker, isolation of the gene(s) will be facilitated.

The RFLP and RAPD markers CT88 and PO9₅₈₇ discussed above can also be used as molecular tags to follow resistance through breeding programs. The use of a combination of the resistance-associated RAPD and RFLP markers can provide an added advantage in following segregation in breeding, and eventually in isolating and cloning the *S. bulbocastanum* gene(s) responsible for conferring resistance to late blight and other diseases.

REFERENCES

Austin, S., M.A. Baer and J.P. Helgeson (1985). Transfer of resistance to potato leaf roll virus from *Solanum brevidens* into *Solanum tuberosum* by somatic fusion. Plant Science 39: 75-82.

Austin, S., E. Lojowska, M.K. Ehlenfeldt, A. Kelman and J.P. Helgeson (1988). Fertile interspecific somatic hybrids of *Solanum*: A novel source of resistance to *Erwinia* soft rot. Phytopathology 78: 1216-1220.

Austin, S., J.D. Pohlman, C.R. Brown, H. Mojtahed, G.S. Santo, D.S. Douches and J.P. Helgeson (1993). Interspecific somatic hybrids between *Solanum tuberosum* and *S. bulbocastanum*: incorporation of nematode resistance. Am. Potato J. 70: 485-495.

Black, W. and M.E. Gallegly (1957). Screening of *Solanum* species for resistance to physiologic races of *P. infestans*. Am. Potato J. 34: 273-281.

Bonierbale, M.W., R.L. Plaisted and S.D. Tanksley (1988). RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120: 1095-1103.

Brown, C.R., C.P. Yang, H. Mojtahedi, G.S. Santo, R. Masuelli (1996). RFLP analysis of resistance to Columbia root-knot nematode derived from *Solanum bulbocastanum* in a BC2 population. Theor. Appl. Genet. 92: 572-576.

- Ehlenfeldt, M.K. and J.P. Helgeson. 1987. Fertility of somatic hybrids from protoplast fusions of *Solanum brevidens* and *S. tuberosum*. Theor. Appl. Genet. 73: 395-402.
- 5 Haberlach G., B.A. Cohen, N.A. Reichert, M.A. Baer, L.E. Towill and J.P. Helgeson (1985). Isolation, culture and regeneration of protoplasts from potato and several related *Solanum* species. Plant Sci 39:67-74.
- Helgeson, J.P., G.J. Hunt, G.T. Haberlach and
10 S. Austin (1986). Somatic hybrids between *Solanum brevidens* and *Solanum tuberosum*: Expression of a late blight resistance gene and potato leaf roll resistance. Plant Cell Reports 3: 212-214.
- Helgeson, J.P., G.T. Haberlach, M.K.
15 Ehlenfeldt, G. Hunt, J.D. Pohlman and S. Austin (1993). Fertile somatic hybrids of potato and wild *Solanum* species: potential for use in breeding programs. Am. Potato. J. 70: 437-452.
- Kim-Lee, H., S.U. Choi, M.S. Chae, S.M. Wielgus
20 and J.P. Helgeson (1993). Identification of somatic hybrids produced by protoplast fusion between *Solanum commersonii* and *S. tuberosum* haploids. Korean Soc. of Plant Tissue Culture 20: 337-344.
- Lam, S.L. (1977). Regeneration of plantlets
25 from single cells in potatoes. Am.Potato J 54:575-580.
- Lander, E.C., P. Green, J. Abrahamson, A. Barlow, M.J. Daly et al. (1987). MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural
30 populations. Genomics 1: 174-181.
- McGrath, J.M., S.M. Wielgus, and J.P. Helgeson (1994). Recombination and introgression of *Solanum brevidens* DNA in progeny of a somatic hybrid with potato. Amer. Potato J. 71: 686-687
- 35 McGrath, J.M., S.M. Wielgus, and J.P. Helgeson (1996). Segregation and recombination of *Solanum brevidens* syntenic groups in progeny of somatic hybrids

with *S. tuberosum*: Intragenomic equals or exceeds intergenomic recombinations. *Genetics* **142**: 1335-1348.

Novy, R.G. and J.P. Helgeson (1994).

Resistance to potato virus Y in somatic hybrids between
5 *Solanum etuberosum* and *S. tuberosum* X *S. berthaultii*
hybrid. *Theor. Appl. Genet.* **89**: 783-786.

Tanksley, S.D., M.W. Ganai, J.P. Prince, M.C.
DeVicente, M.W. Bonnierbale et al. (1992). High density
molecular linkage maps of the tomato and potato genomes.
10 *Genetics* **132**: 1141-1160.

Williams, C.E., G. Hunt, and J.P. Helgeson
(1990). Fertile somatic hybrids of *Solanum* species: RFLP
analysis of hybrid and its sexual progeny from crosses
with potato. *Theor. Appl. Genet.* **80**: 545-551.

15 Williams, J.G.K., A.R. Kubelik, K.J. Liwak,
J.A. Rafalsi and S.V. Tingley (1990). DNA polymorphisms
amplified by arbitrary primers are useful as genetic
markers. *Nucl. Acids Res.* **18**: 6531-6535.

20 The present invention is not limited to the
embodiments described and exemplified above, but is
capable of variation and modification without departure
from the scope of the appended claims.

What is claimed is:

1. A late blight-resistant potato plant comprising a segment of a genome from *Solanum bulbocastanum* which comprises a gene that confers said resistance to late blight.

5
2. The potato plant of claim 1, wherein the segment of the *Solanum bulbocastanum* genome is a segment of chromosome 8 of the genome.

10
3. The potato plant of claim 1, wherein the gene conferring the late blight resistance co-segregates with a marker selected from the group consisting of a GO2₅₈₆ RAPD marker, a PO9₅₈₇ RAPD marker, a CT88 RFLP marker, a CT148 RFLP marker, a CT252 RFLP marker and a CT68 RFLP marker.

15
4. The potato plant of claim 4 wherein the marker comprises a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 AND SEQ ID NO:5.

20
5. The potato plant of claim 1, which is also resistant to at least one additional disease selected from the group consisting of potato early blight, *Erwinia* soft rot, and *Verticillium* wilt.

25
6. The potato plant of claim 1, wherein the late blight resistance gene is incorporated into the plant by somatic hybridization between a cell of a parent of the plant and a cell of *Solanum bulbocastanum*.

30
7. The potato plant of claim 1, wherein the late blight resistance gene is incorporated into the plant by genetic transformation of a cell of the plant with a plant transforming vector comprising the gene.

35

8. An isolated nucleic acid molecule which is complementary to part or all of a double-stranded molecule selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:5.

5

9. The nucleic acid molecule of claim 8, disposed within a vector.

10. A method of monitoring late blight resistance in a breeding cross of progeny of a fertile somatic hybrid of *Solanum tuberosum* and *Solanum bulbocastanum*, which comprises:

(a) performing the cross;

(b) isolating genomic DNA from individual progeny of the cross; and

(c) detecting in the genomic DNA the presence or absence of a genetic marker that is pre-determined to co-segregate with the late blight resistance, the presence or absence of the marker being indicative of the presence or absence of the late blight resistance in the individual progeny of the breeding cross.

11. The method of claim 10, wherein the marker is selected from the group consisting of a GO2₅₈₆ RAPD marker, a PO9₅₈₇ RAPD marker, a CT88 RFLP marker, a CT148 RFLP marker, a CT252 RFLP marker and a CT68 RFLP marker.

12. A method of identifying a *Solanum bulbocastanum* gene that confers resistance to late blight, said method comprising:

(a) cloning a DNA segment that co-segregates with the late blight resistance phenotype in progeny of somatic hybrids of *Solanum bulbocastanum* and *Solanum tuberosum*;

(b) providing a genomic library of the *Solanum bulbocastanum* genome;

(c) isolating clones of the genomic library that contain segments which hybridize with the co-segregating DNA segment; and

5 (d) identifying at least one gene disposed within the isolated genomic clones that confer the late blight resistance.

13. The method of claim 12, wherein said cloned DNA segment that co-segregates with late blight
10 resistance comprises part or all of a marker selected from the group consisting of a G02₅₈₆ RAPD marker, a PO9₅₈₇ RAPD marker, a CT88 RFLP marker, a CT148 RFLP marker, a CT252 RFLP marker and a CT68 RFLP marker.

15 14. A late blight resistance gene from *Solanum bulbocastanum*, produced by the method of claim 12.

15. A transgenic plant comprising the gene of claim 14.

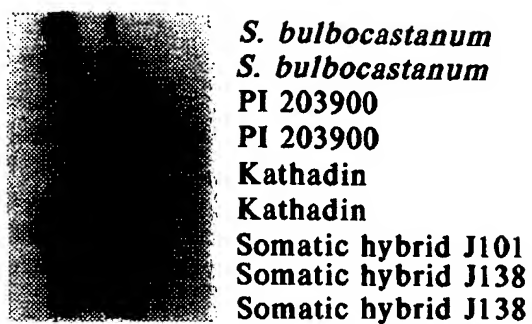
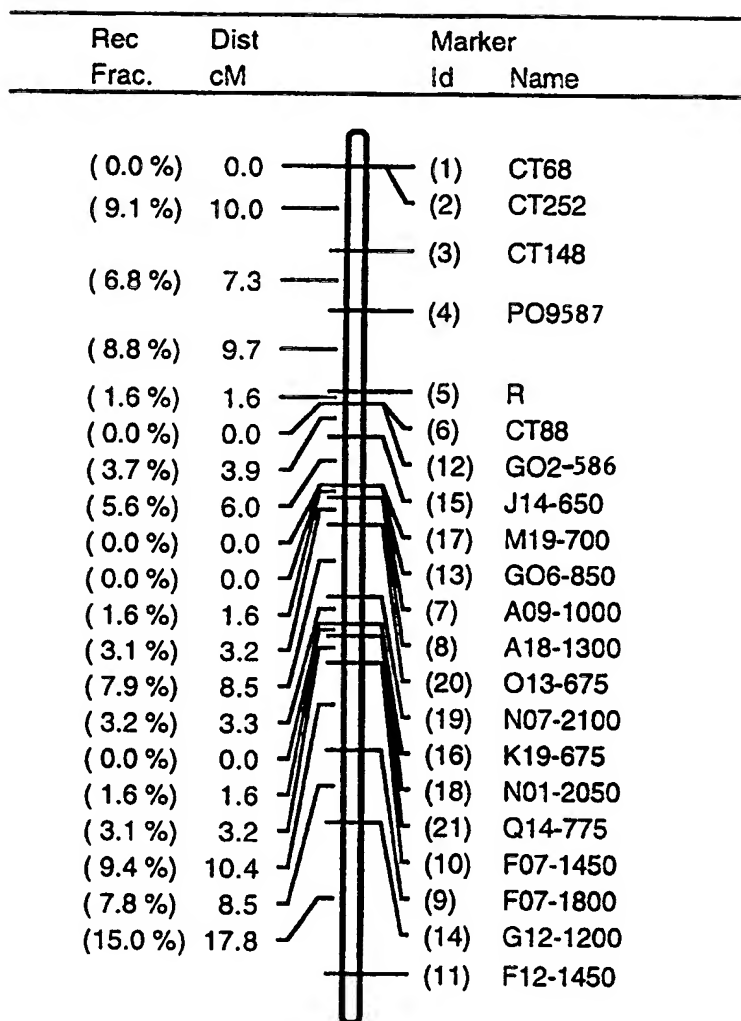


FIG. 1

2/3

**Figure 2**

3/3

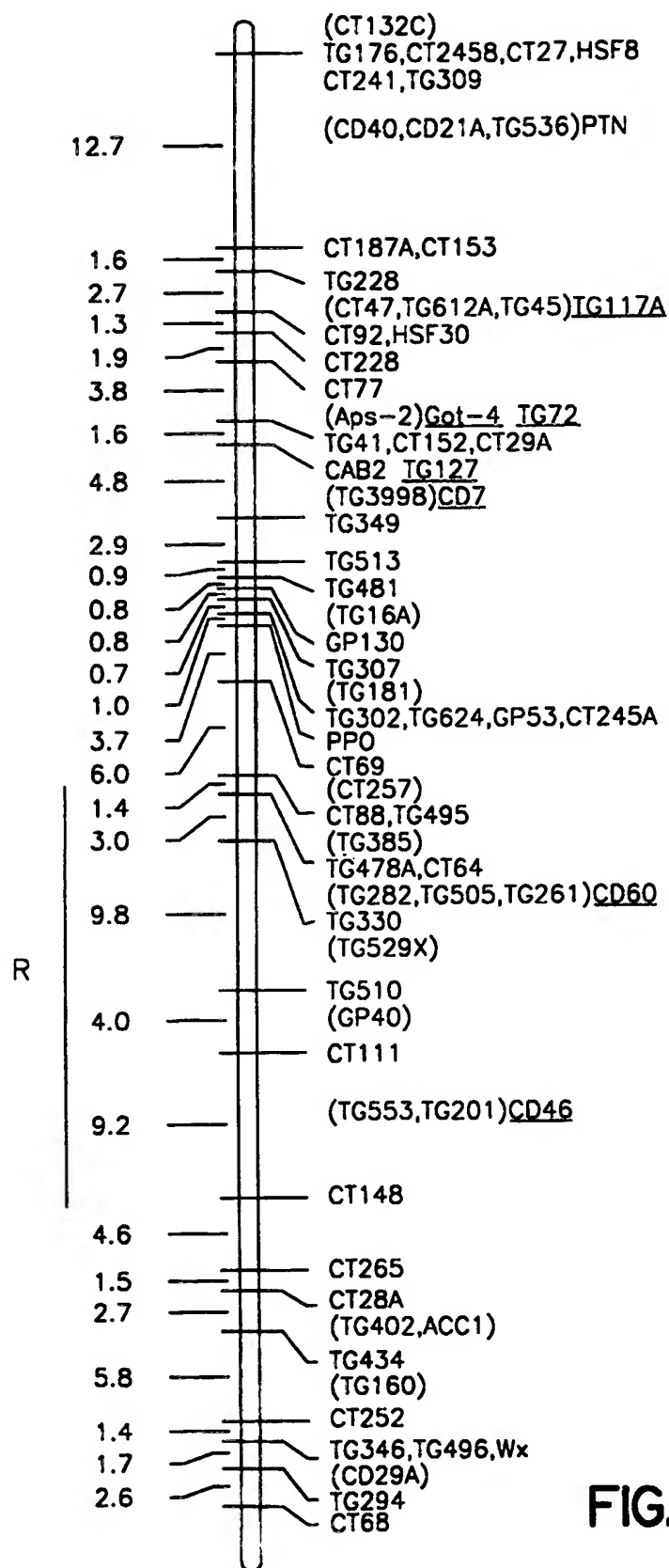


FIG. 3

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Helgeson, John P.
Austin, Sandra
Naess, Sara K.
- 10 (ii) TITLE OF THE INVENTION: GERMPLASM AND MOLECULAR
MARKERS FOR DISEASE RESISTANCE IN POTATO
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
15 (A) ADDRESSEE: Dann, Dorfman, Herrell & Skillman
(B) STREET: 1601 Market Street, Suite 720
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: USA
20 (F) ZIP: 19103
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
25 (C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 1.5
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Not yet assigned
30 (B) FILING DATE: 27-JUL-1998
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/054,267
(B) FILING DATE: 30-JUL-1997
35
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Janet E. Reed
(B) REGISTRATION NUMBER: 36,252
(C) REFERENCE/DOCKET NUMBER: P98003WO
40
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-563-4100
(B) TELEFAX: 215-563-4044
- 45 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 586 base pairs
(B) TYPE: nucleic acid
50 (C) STRANDEDNESS: double
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
55 (v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: Solanum bulbocastanum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

	GGCACTGAGG	GGTAGTAAGC	CTCCTGCATG	TACTAAGTAT	GGTAGATCCA	CTCAGGGTTG	60
	TGCCATGATG	GCTTAACTGG	TTGTTTCAAG	TATGGCCAGA	ACGGTTATTT	TATGAGAGAG	120
5	TTTCCAAAGA	ACATGCAGGG	TAATGGTAAT	GGGGATAATA	GAACCCAGTC	TTCTTCAGTG	180
	ACTCCACCAG	ACAGAGCTGC	ATCTAGAGGA	GCTACTTCGA	GGCAGGCGGA	GGATCGAACC	240
	TCTTTATGCT	ATCACTAGTC	GCCAAGAGAA	AGAGGATTCG	CCAGATGTTG	TCACTGGTAT	300
	GATCCAAGTC	TTTAACTTG	ATTTTATACT	TTTCTAGATC	CAGGAGCGAG	TTTATCCTTT	360
	GTAACCTCCT	ATGTTGCGGT	TAATTTTGAT	GTTCTTCCTA	AGAAACTTAT	TGAGCCCTTC	420
10	AGTGTTTCTA	CACTTGTTGG	TCTATTATAG	TAGAGAGAGT	CTGTTATGAT	TGTACCGTTT	480
	TCGTCAATCA	CAAGAGCACC	ATGGTTGATT	TAGTTGAGTT	AGACATGGTA	GAATTTGATG	540
	TTATTCTTGG	TATGGACTGA	CTTCATTCTT	GTTATGCCTC	AGTGCC		586

(2) INFORMATION FOR SEQ ID NO:2:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 587 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

20

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

25

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE: Solanum bulbocastanum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

30	GTGGTCCGCA	TATAACTCAA	GAAGTTGTAA	TGCATGTATC	GGATATATGT	ATACATGTTG	60
	TCTTTTGCAA	AGTTTACTTT	TTTATTANTT	AATCTTGTTT	GTGTCTGGAG	GTGGTGGTGG	120
	GGTGGGATAG	TGGTGAAGCT	AGAAATTTAG	TTAAGTGTGT	TCAAGATTTA	AATATACATA	180
	TGAAAAATAA	TTTTTGATCT	ATATATATAG	TTATAATTTT	NTGATGAAGG	TAGTTCAACT	240
	GACCACCCGT	NACTACATGT	GGCTACCGTA	CTGGGTGGGG	TGGAAGGTCN	TGGTGTTTAC	300
35	AGTGATGTGG	GGGCCCTCTGA	AATGCTTTTG	TGGGCAATGT	GGGAATTACT	GTTTATCTTT	360
	TCTTTATTGA	AGTCATTGAG	TGTTTGAGTT	ATTTAACTAT	GAAAGGTAGC	TAGTGGGTAA	420
	TGTTATTGAT	GACTTTGTGT	GAAGAACAAA	ATGTCAATCA	TTCAGAGCGT	TCAATGGGGG	480
	CACGTGCTGG	AGTCCCATTT	GGATTATTGG	GTTTAGGGGT	TGCCTAGGTG	GTGGTGGTGG	540
40	TGTTTGGTAA	TGGATAATAA	TGTTGGTGAA	ATATGGGTGC	GGAGGAG		587

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 596 base pairs

45

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

50

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE: tomato

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

	GTGGGGCAGA	AGAGCTAGGA	AGAGTAAGCA	TGTCAAGTGA	TAGTTGCAGC	CACTGGTGTG	60
	ATAGTTGTAG	ACAACCCGTG	AATCTCAGGA	GACAAAATGA	TGTTTGCCCC	AATTGCGGTG	120
	GTGGATTTGT	TCAAGAGCTT	GAAGACATAA	CGAGTAGTAG	TGTAGATAAT	CAGACCCAGA	180
60	GGCCGAGATT	CATGGAAATC	GTCTCAAACT	TTTTAAGACG	ACAAATCTCA	GCTACAAGTA	240
	ATACTTCTGA	GAGAGGGAGA	TCTGATGGGG	GTGCTGAACG	AGGAAATTTG	TGGAATCCGT	300

	TGCTGATTTT	CAGTGGTGAT	ACGCCTGTTT	ATATGCCTGG	GGATGGTGGG	GTTTTGGAGT	360
	TTCTTAATGA	GGCACCTTGG	ATTCCGAGCA	AGAAAAATGGT	GGTGATTATT	TTGTTGGTCC	420
	AGGAGTGGAG	GAATTTTTTG	AAGAAATTGT	AAATAGAAAT	CAGCGTGGTG	CTCCTCCTCC	480
	TGTCTCGAGA	TGTTCAATTG	ATTCCCTACC	AACAGTCAAG	ATATCAAAAA	AGGATGTTAG	540
5	ATCGGATTCT	CACTGCCCTG	TTTGTAAGGA	GAAATTTGCT	CTGGGGACTA	AGGCAA	596

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- 10 (A) LENGTH: 589 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
- 15 (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
(v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: Solanum tuberosum
- 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

	GTGGGGCAGA	AGAGCTAGGA	AGAGTAAGCA	TGTCAAGTGA	CAGTTGCAGC	CACTGGTGTG	60
	ATAGTTGTAG	ACAACCCGTG	AATCTCAGGA	GACAAAATGA	TGTTTGCCCC	AATTGCAGTG	120
25	GTGGATTTGT	TCAAGAGCTT	GAAGACATAA	CGAGTAGTAG	TGTAGATAAT	CAGAGCCAGA	180
	GGCCGAGATT	TATGGAATCC	GTCTCAAACT	TTTTAAGACG	ACAAATCGCT	ACAAGTAATA	240
	CTTCTGAGAG	AGGGAGATCT	GATGGGGGTG	CTGAACGAGG	AAATTTATGG	AATCCATTGC	300
	TGATTTTCAG	TGGTGATACG	CCTGTTCAAG	ATGCCTGGGG	ATGGTGGAGT	TTTGGAGTTT	360
	CTTAATGAGG	CTCTTGGCTT	CCGACAAGAA	AATGGTGGTG	ATTATTTTGT	TGGTCCAGGA	420
30	GTGGAGGAAT	TTTTTGAAGA	AATTGTAAAT	AGAAATCAGC	GTGGTGCTCC	TCCTGCCTCA	480
	AGATGTTCAA	TTGATTCCCT	ACCAACAGTC	AAGATATCGA	AAAAAGATGT	TAGATCGGAT	540
	TCTCACTGCC	CTGTTTGTA	AGAGAAATTT	GCTCTGGGGA	CTAAGGCAA		589

(2) INFORMATION FOR SEQ ID NO:5:

- 35 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 592 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
40 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTISENSE: NO
45 (v) FRAGMENT TYPE:
(vi) ORIGINAL SOURCE: Solanum bulbocastanum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

50	GTGGGGCAGA	AGAGCTAGGA	AGAGTAAGCA	TGTCAAGTGA	CAGTTGCAGC	CACTGGTGTG	60
	ATAGTTGTAG	ACAACCCGTG	AATCTCAGCA	GACAAAATGA	TGTTTGCCCC	AATTGCGGTG	120
	GTGGATTTGT	TCAAGAGCTT	GAAGACATAA	CGAGTAGTAG	TGTAGATAAT	CAGAGCCAGA	180
	GGCCGAGATT	CATGGAATCC	GTCTCAAACT	TTTTAAGACG	ACAAATCCCA	ACTACAAGTA	240
	ATACTTCTTG	AGAGAGGGAG	ATCTGATGGG	GGTGCTGAAC	GAGGAAATTT	GTGGAATCCG	300
55	TTGCTGATTT	TCAGTGGTGA	TACACCTGTT	CGGATGCCTG	GGGATGGTGG	AGTTTTGGAG	360
	TTTCTTAATG	AGGCTCTTGG	CTTTCGACAA	GAAATATGGT	GTGANTATTT	TGTTGGCCCA	420
	GGAGTGGAGG	AGTTTTTTGA	AGAAATTGTA	AATAAAATC	AGCGTGGTGC	TCCTCCTGTC	480
	TCAAGATGCT	CAATTGATTC	CCTACCAACA	GTCAAGATAT	CGAAAAAGGA	TGTTAGATCG	540
	GATTCTCACT	CCCCTGTTTG	TAAAGAGAAA	TTTGCTCTGG	GGACTAAGGC	AA	592

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15910**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A01H 5/00, 1/00, 1/04; C12N 15/05, 15/09, 15/11

US CL :800/317.2, 298, 301, 265, 267, 277, 279; 435/468; 536/23.1, 23.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/317.2, 298, 301, 265, 267, 277, 279; 435/468; 536/23.1, 23.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CABA, AGRICOLA, WPINDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ---	HELGESON et al. Somatic hybrids between Solanum bulbocastanum and potato: a new source of resistance to late blight.	1,2,5,6,10 -----
Y, P	Theoretical and Applied Genetics. 1998. pp. 738-742, see entire article.	3,4,7-9,11-15
X --	THIEME et al. Production of somatic hybrids between S tuberosum L. and late blight resistant Mexican wild potato species. Euphytica.	1,2,5,6,10 -----
Y	1997. Vol. 97. Number 2, pp. 189-200, see entire article.	3,4,7-9,11-15
X --	SCHUMANN et al. Somatic hybridization of Solanum tuberosum L. with Phythphora-resistant wild potato species. Physiologia Plantarum. 1991. Vol. 82. pp. A23, abstract 134, see entire abstract.	1,5,6 -----
Y		2,10

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 OCTOBER 1998

Date of mailing of the international search report

03 NOV 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer
MELISSA KIMBALL

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15910

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,750,874 A (STRITTMATTER et al.) 12 May 1998, example 3, col. 16-18.	1,7,12-15
A	MASUELLI et al. Irregular meiosis in a somatic hybrid between <i>S. bulbocastanum</i> and <i>S. tuberosum</i> detected by species-specific PCR markers and cytological analysis. Theoretical and Applied Genetics. 1995. Vol. 91. pp. 401-408, see especially page 401, col. 1 and page 402, col. 2.	1-15